

# Laboratory Technology Developments Sensitive Fluorescent Stains for Detecting Nucleic Acids in

## Gels and Solutions

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Three new classes of unsymmetrical cyanine nucleic acid stains have been developed for the sensitive detection of nucleic acids in a wide variety of bioanalytical applications. These dyes have negligible intrinsic fluorescence but large fluorescence yields when bound to DNA and/or RNA. Several of the new dyes are particularly useful for quantitating nucleic acids in electrophoretic gels and in solution, providing sensitivity limits that are orders-of-magnitude greater than those achieved with conventional dyes. Furthermore, assays employing the new dyes readily lend themselves to automation and high-throughput analysis because of their ease of application and because of their compatibility with a wide

### Introduction

Due to increases in both the total number of commercially available drugs made from recombinant or genetically modified biological sources and in the number of federal guidelines governing manufacture of such products, sensitive methods for detecting and quantitating small amounts of nucleic acids have become essential to the biotechnology industry. In addition, the current large scale effort to identify and characterize genes involved in complex human diseases, such as asthma, schizophrenia and diabetes, has required reliable quantitation of small amounts of DNA to be isolated from human donors prior to further analysis. Detecting and analyzing microbial cells present in environmental samples, such as potable water, sewage treatment plant effluents, and seawater, also often requires sensitive methods to quantitate DNA yields prior to species identification analysis. Although a number of sensitive multi-step procedures have been developed for these purposes, including polymerase chain reaction (PCR) and other hybridization- or DNA capture based target amplification techniques, the simplest methods remain those that employ dyes or stains that become fluorescent only when bound to nucleic acids. We describe here a number of new unsymmetrical cyanine dyes with this property, which are extremely sensitive reagents for detecting nucleic acids in gels and in solutions.

variety of commercially available detection instrumentation.

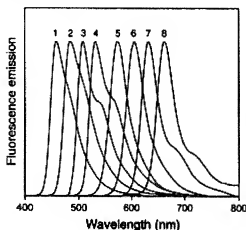


Figure 1. Normalized fluorescence emission spectra of the unsymmetrical cyanine dimer dyes, bound to ds calf thymus DNA. Peaks correspond to the emissions of 1) POPO-1, 2) BOBO-1, 3) YOYO-1, 4) TOTO-1, 5) POPO-3, 6) BOBO-3, 7) YOYO-3, and 8) TOTO-3.

## Sensitivity Targets

Although sensitive assays for detecting and quantitating nucleic acids are needed in a wide variety of bioanalytical fields, the level of sensitivity that is required varies with the specific application. In environmental microbiology and soil science, for example, because many of the organisms present in environmental samples are not readily culturable and because complex mixtures of organisms are usually present, it is frequently necessary to analyze these organisms after extraction of their DNA or RNA.<sup>1</sup> However, yields of bacterial DNA from the soil are relatively high-milligram levels of DNA can be obtained from ~200 g of soil and sample sizes are generally not limiting.<sup>2</sup> In contrast, levels of microbial or viral nucleic acids in drinking water or in sewage treatment effluents are much lower and often require more sensitive detection methods. For forensic analysis, blood samples or buccal scrapes generally yield only about 1-10 ng DNA. Although it has been possible to do genetic analysis on single human hairs, samples containing less than nanogram levels of DNA are generally not considered to be suitable for short tandem repeat (STR), restriction fragment length polymorphism (RFLP) or other analysis. Thus, the ability to reliably quantitate 100 pg quantities of DNA would be sufficient for most purposes.

Perhaps the most stringent DNA sensitivity requirements come from the pharmaceutical field. Genomic DNA per se, which might be retained as an impurity in biopharmaceutical preparations, has been shown to not provide significant risk to the recipient of the pharmaceutical agent.<sup>3</sup> However, there is still concern about the presence of potentially hazardous DNA species, such as those encoding viral proteins or oncogene products.

The dosage of DNA that is considered to be "safe" is 100 pg DNA per dose of a pharmaceutical agent.<sup>4</sup> As Briggs and Panfili explain,<sup>5</sup> this number comes from a calculation extrapolated from tumor-injection studies as follows: a dose of 2  $\mu$ g of oncogenic virus DNA was found to be a "tumor-inducing dose" because it induced tumors in ~50% of the injected animals. If the chromosomal DNA is assumed to have incorporated only one copy of the oncogene per genome, the oncogene represents about  $10^{-6}$  of the total cellular DNA. Thus, a dose of a drug containing 100 pg of genomic DNA contains only ~0.1 fg of oncogene DNA. The risk from this small amount of DNA was considered to be essentially

negligible.<sup>6</sup> However, the FDA recommends that any detection "method ought to provide sensitivity on the order of 10 picograms per dose."<sup>7</sup> Neither WHO nor FDA recommendations specify whether this DNA should be analyzed in solution, by gel or capillary electrophoresis, or by other means.

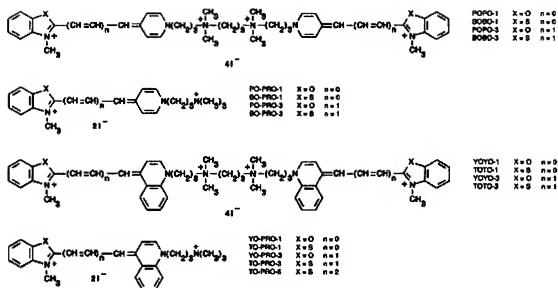


Figure 2. Structures of some of the unsymmetrical cyanine monomers and dimers.

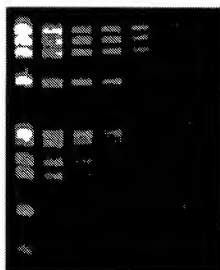


Figure 3.1 Polyacrylamide gels containing ds DNA stained with PO-PRO-1

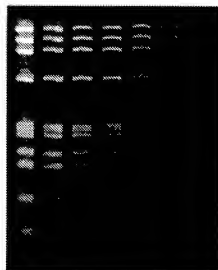
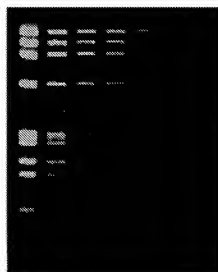


Figure 3.2 BO-PRO-1



Figure 3.3 YO-PRO-1



TO-PRO-1

Lanes contain identical threefold dilution series of bacteriophage fX174 RF DNA cut with *Hae*III restriction endonuclease: 1) 3  $\mu$ g, 2) 1  $\mu$ g, 3) 360 ng, 4) 120 ng, 5) 40 ng, 6) 13.5 ng, and 7) 4.5 ng total DNA per lane. Gels were stained with 1  $\mu$ M of each dye in 89 mM Tris base, 89 mM boric acid, 1 mM

EDTA, pH 8.0, then photographed using 254 nm epi-illumination, through a 410 nm longpass glass filter (Oriel), with a FOTO/UV® 450 transilluminator (FotoDyne) and Ektachrome 400 Elite color slide film.

## Detection Reagents

Although hybridization, DNA capture, target amplification, and signal amplification based assays for nucleic acids are extremely sensitive, they are also time-consuming and comprise multiple steps; thus whether the detection step utilizes radioactivity, chemiluminescence, or fluorescence, these assays are not ideally suited to high-throughput analysis. In addition, the sample manipulation steps required for these procedures introduce potential sources of error and analyte contamination. One of the greatest advantages provided by sensitive, fluorogenic nucleic acid stains-dyes that become fluorescent only upon binding nucleic acids-is that they provide one-step methods with single, short incubation times. These methods are readily automatable and well suited to high-throughput analysis.

Fluorogenic nucleic acid stains have been available for many years. The use of the intercalating dye ethidium bromide for quantitating nucleic acids in solution was described in the mid-1960s.<sup>8,9</sup> Ethidium bromide provides a sensitivity for double-stranded (ds) DNA detection ( $\sim 10$  ng/mL) considerably greater than that provided by ultraviolet absorbance measurements ( $\sim 1$   $\mu$ g/mL), with greater selectivity for nucleic acids over proteins. The use of ethidium bromide to detect nucleic acids in slab gels was first described in the scientific literature more than 20 years ago.<sup>10</sup> It is still the most commonly used dye in that application. However, ethidium bromide also exhibits significant fluorescence upon binding RNA and single-stranded (ss) DNA,<sup>8,9</sup> and has a narrow dynamic assay range for a given dye concentration.<sup>11</sup> Although it did not provide greater assay sensitivity, the bis-benzimide dye Hoechst 33258 was shown to be superior to ethidium bromide for solution quantitation because of its greater selectivity for ds DNA over RNA and ss DNA.<sup>12</sup> However, Hoechst 33258 dye is very AT-selective in its fluorescence,<sup>13-17</sup> has a limited dynamic assay range for a given dye concentration, and requires two different salt concentrations to differentiate ds DNA from ss DNA or from RNA.<sup>12</sup> Ethidium homodimer-1, a dimer

of ethidium, has been shown to allow detection of as little as 1 ng/mL DNA.<sup>18</sup> However, both RNA and ss DNA interfere with the assay and it also has a narrow dynamic range.

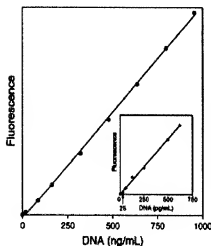


Figure 4. Sensitivity, linearity and dynamic range of detection of ds DNA using PicoGreen reagent. Calf thymus DNA was diluted in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA (T.E.) in 1 mL volumes. The stock solution of PicoGreen reagent was diluted 1:200, to a final concentration of 1.6  $\mu$ M, in T.E. and 1 mL was added to each DNA aliquot. Samples were incubated for 5 minutes at room temperature, protected from light, and fluorescence measured at 520 nm after excitation at 485 nm, using a spectrofluorometer. Fluorescence intensity was integrated and plotted versus DNA concentration.

## Characteristics of the New Dyes

Over the last 6 years, Molecular Probes has sought to improve upon existing fluorogenic nucleic acid stains and has done so by developing a new series of unsymmetrical cyanine dyes with useful properties. The new dyes can be divided into three chemical classes: monomer dyes with cationic side chains,<sup>19</sup> dimers of these dyes,<sup>20</sup> and substituted monomers.<sup>21</sup> All of these dyes have significant advantages over conventional nucleic acid stains, including high quantum yields (some as high as 0.8-0.9), large molar extinction coefficients ( $\sim 50,000$ - $80,000$   $\text{cm}^{-1}\text{M}^{-1}$ ), good DNA binding affinities, extremely large fluorescence enhancements upon binding nucleic acids (some  $>1000$ -fold), and negligible fluorescence for the free dyes.<sup>22</sup> In addition, these dyes have fluorescence excitations and emissions that span the entire visible spectrum (Fig. 1). Dyes in this series were specifically designed to have fluorescence excitation spectra that overlap most laser lines and both ultraviolet and visible maxima of other, commonly used light sources. Examples of representative structures of the first two dye classes are shown in Figure 2. The monomer dyes with cationic side chains were found to penetrate electrophoretic gels reasonably rapidly, providing sensitive, multicolor stains for this purpose (Fig. 3).<sup>23</sup> They have also been used for quantitating ds DNA in solution,<sup>24</sup> for detecting DNA separated by capillary electrophoresis,<sup>25</sup> and for staining cells,<sup>26-28</sup> tissues,<sup>29</sup> and viruses.<sup>30,31</sup> The dimeric cyanine dyes have extremely high affinities; this property has proven useful in a variety of applications. They have been used as electrophoretic prestains,<sup>32,34</sup> as labels for detecting single DNA molecules by fluorescence microscopy<sup>35,36</sup> and in fluid streams,<sup>37</sup> for detecting DNA separated by capillary electrophoresis,<sup>38-40</sup> and for staining chromosomes,<sup>41</sup> nuclei,<sup>41</sup> cells,<sup>26,28,41,42</sup> and viruses.<sup>30</sup> These dyes have also been employed for quantitating ds DNA,<sup>24,43,44</sup> oligonucleotides,<sup>45</sup> and nucleases<sup>46</sup> in solution. Although these dimer dyes are extremely bright, they have only a narrow linear range for nucleic acid quantitation in solution<sup>24,43</sup> and penetrate electrophoretic gels very slowly.

In efforts to improve upon these dyes, we found that the introduction of specific substituents to particular positions on the unsymmetrical cyanine core molecule resulted in the creation of a new class of nucleic acid stains,<sup>47</sup> some of which have quantum yields much higher than those of the best of the previous dyes. SYBR® Green I nucleic acid gel stain, for example, has a quantum yield on binding ds DNA of  $\sim 0.8$ .<sup>48</sup> YOYO®-1, in comparison, is the brightest of the dimer dyes and has a fluorescence quantum yield of  $\sim 0.5$  when bound to ds DNA,<sup>49</sup> whereas ethidium bromide has a fluorescence quantum yield of only 0.15 when bound to ds DNA. By modifying these new dyes, we were able to adjust their

affinities to yield dyes with extremely broad dynamic assay ranges for quantitating DNA in solution, to create dyes that have extremely high quantum yields on binding ss DNAs-including short oligonucleotides-and to generate dyes that bind to RNA with good fluorescence enhancements. The characteristics of four of these new dyes, PicoGreen® dsDNA quantitation reagent, OliGreen® ssDNA quantitation reagent, SYBR Green I nucleic acid gel stain, and SYBR Green II RNA gel stain, are described in detail below.

Table 1

### Comparison of Dyes For Quantitating Nucleic Acids in Solution

Dye/Method	Dye Chemical	Excitation Maximum/ Emission Maximum*	Sensitivity Limit <sup>†</sup>	Comments
UV absorbance	none	260 nm absorbance	~1 µg/mL	RNA, free NTPs, proteins, some amino acids interfere
ethidium bromide	phenanthridinium monomer	518 nm/605 nm	10 ng/mL <sup>1</sup>	RNA interferes; high intrinsic dye fluorescence; several dye concentrations needed for full assay dynamic range
Hoechst 33258	bis-benzimide	352 nm/461 nm	10 ng/mL <sup>2</sup>	AT-selective; requires high salt to differentiate dsDNA from RNA and low salt to differentiate dsDNA from ssDNA; two dye concentrations needed for full assay dynamic range
YO-PRO-1	unsymmetrical cyanine monomer	491 nm/509 nm	2.5ng/mL <sup>3</sup>	oligonucleotides and RNA interfere
ethidium homodimer-1	phenanthridinium dimer	528 nm/617 nm	1ng/mL <sup>4</sup>	oligonucleotides and RNA interfere; narrow dynamic range
YOYO-1	unsymmetrical cyanine dimer	491 nm/509 nm	0.5ng/mL <sup>5</sup>	narrow dynamic assay range; slow binding rate
PicoGreen	substituted unsymmetrical cyanine monomer	502 nm/523 nm	25 pg/mL <sup>6</sup>	broad dynamic assay range with single dye concentration; highly selective for ds DNA; not AT/GC selective

\* Wavelengths indicated are for the dye bound to double-stranded DNA.

<sup>†</sup> Sensitivity Limit indicates the limiting concentration for reliably quantitating double-stranded DNA in a standard 10 mm spectrofluorometer cuvette, under optimal assay conditions for the dye.

<sup>‡</sup> The sensitivity limit reported for YO-PRO-1 was obtained using a fluorescence microplate reader.

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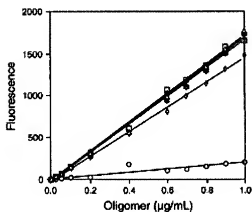
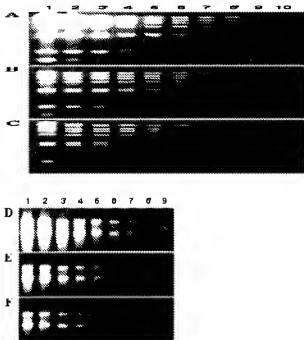


Figure 5. Insensitivity of the OliGreen assay to the size of the ss DNA. The indicated concentrations of random sequence oligodeoxynucleotides were incubated with 0.5  $\mu$ M OliGreen reagent in T.E. (400-fold dilution of the stock solution) for 5 minutes, then fluorescence was measured at 530 nm, after excitation at 485 nm, using a fluorescence microplate reader. Fluorescence intensity was integrated and plotted versus DNA concentration. Samples were 6-mers (open circles), 10-mers (open diamonds), 20-mers (open triangles), 30mers (open stars), 40-mers (closed stars), and 50-mers (open squares).



## Solution Quantitation

We screened the new substituted cyanine dyes to find those that provided optimal quantitation of ds DNA in solution and of ss DNA (and ss oligodeoxynucleotides) in solution. PicoGreen dsDNA quantitation reagent was found to allow detection of as little as 25 pg/mL ds DNA using 2 mL assay volumes and standard 10 mm cuvettes in a fluorometer (Fig. 4), or 250 pg/mL using 200  $\mu$ L assay volumes and a fluorescence microplate reader (data not shown). This sensitivity is 400 times greater than that reported for Hoechst 33258 and more than 10 times greater than that achieved with the monomeric or dimeric cyanine dyes discussed above (Table 1). The data are extremely linear ( $r^2$  values greater than 0.998) and span more than four orders of magnitude in DNA concentration using a single dye concentration. The PicoGreen assay not only shows greater sensitivity for detecting ds DNA than the reagents described above, but also has greater selectivity.<sup>50</sup> The reagent is also not base-selective; quantum yields and fluorescence enhancements of PicoGreen dye bound to poly(dA) poly(dT) are the same as those obtained with dye bound to poly(dG) poly(dC).<sup>50</sup> Proteins, nucleotides and other contaminants commonly found in DNA preparations do not appreciably interfere with the PicoGreen assay.<sup>50</sup> The assay procedure is simple: samples are diluted in T.E. buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) to an appropriate volume. An equal volume of a 1:200 dilution of PicoGreen reagent is added to the sample. The mixture is allowed to incubate for 5 minutes, then fluorescence is measured directly. The dye has an excitation maximum at  $\sim$ 500 nm and an emission

Figure 6. Sensitivities of SYBR Green I stain for detecting ds DNA and for SYBR Green II stain for detecting RNA. Identical twofold dilutions of bacteriophage  $\lambda$ 1857 DNA digested with HindIII restriction endonuclease were separated on 1% agarose gels and stained for 20 minutes with a 1:10,000 dilution of SYBR Green I nucleic acid gel stain (A,B), or with 0.5  $\mu$ g/mL ethidium bromide and then destained in water for an additional 20 minutes (C). Lanes contain 1) 490 ng, 2) 160 ng, 3) 55 ng, 4) 18 ng, 5) 6.1 ng, 6) 2 ng, 7) 0.68 ng, 8) 0.23 ng, 9) 0.075 ng, and 10) 0.025 ng total DNA (A C). Identical twofold dilutions of *E. coli* rRNA were separated on 1% agarose gels and stained for 20 minutes with a 1:10,000 dilution of SYBR Green II stain (D,E) or with 5  $\mu$ g/mL ethidium bromide and then destained for an additional 20 minutes with water (F). Lanes contain 1) 77 ng, 2) 38 ng, 3) 19 ng, 4) 9.6 ng, 5) 4.8 ng, 6) 2.4 ng, 7) 1.2 ng, 8) 0.6 ng, and 9) 0.3 ng total RNA (D F). Gels were photographed through a SYBR Green gel photographic filter (A,B,D,E) or an ethidium bromide photographic filter (C,F), after 254 nm epi-illumination (A,D) or 300 nm transillumination (B,C,E,F), using Polaroid 667 black-and-white print film.

maximum at ~520 nm when bound to nucleic acids, so it is compatible with standard fluorescein filter sets or instrument settings.

PicoGreen reagent has already proven useful in a number of applications. It has been used to quantitate ds DNA templates prior to PCR51 and DNA amplification products resulting from PCR reactions.<sup>52,53</sup> Taq DNA polymerase, free deoxynucleotides and primer pairs do not interfere with these measurements.<sup>51</sup> Because of the simplicity of the assay format, PicoGreen reagent has been successfully used to quantitate DNA for high throughput genotyping experiments.<sup>54</sup> The ds DNA selectivity of the PicoGreen assay formed the basis of solution assays developed for detecting and quantitating mammalian telomerase,<sup>55</sup> *Escherichia coli* DNA polymerase III holoenzyme,<sup>56</sup> and human immunodeficiency virus reverse transcriptase<sup>56</sup> activities. PicoGreen reagent also compared favorably with both the monomeric and dimeric cyanine dyes described above for enumerating prokaryotic cells by flow cytometry.<sup>28</sup>

While the PicoGreen assay is both sensitive and selective for detecting ds DNA, OliGreen reagent provides a sensitive method for quantitating ss DNA. As little as 100 pg/mL ss DNA or oligodeoxynucleotides can be detected using 2 mL assay volumes and a fluorometer, and as little as 1 ng/mL ss DNA can be detected using 200  $\mu$ L assay volumes in a fluorescence microplate reader.<sup>22</sup>

This sensitivity is approximately 10,000 times that achieved with ultraviolet absorbance measurements. Oligonucleotides are generally difficult to detect in solution due to their lack of secondary structure. Intercalators such as ethidium bromide and groove binding dyes such as the Hoechst dyes bind these molecules very poorly. In contrast, the fluorescence response of the OliGreen assay is independent of nucleic acid size for oligonucleotides greater than 10 bases in length (Fig. 5). OliGreen reagent does not appear to bind well to very small oligonucleotides such as hexamers. Large ss DNAs, such as bacteriophage M13 or X174 DNAs, are also detected with good sensitivity; thus the dye can be used for



quantitating yields of phage preps prior to sequencing, or for quantitating genomic DNA isolated using procedures resulting in denatured DNA.<sup>57</sup> The OliGreen reagent has also been employed to develop a method for detecting ss oligodeoxynucleotides by capillary gel electrophoresis with laser-induced fluorescence, providing much greater sensitivity than can be achieved with ultraviolet absorbance measurements.<sup>58</sup>

We found that the OliGreen reagent can be used to detect oligonucleotides in complex mixtures, such as blood or serum, with good sensitivity.<sup>59</sup> Extraction of the serum with phenol and chloroform reduced background fluorescence but did not affect assay sensitivity, linearity, or dynamic range. OliGreen reagent does exhibit base-selectivity in its fluorescence,<sup>22</sup> however, so the best results are achieved when oligonucleotides with similar base compositions to those under analysis are used to generate the assay standard curve. Much greater signals are observed with oligo (dT) homopolymers than with oligo (dA), d(C), or d(G) homopolymers; the highest signals are obtained with oligonucleotides containing mixed base content. This effect is even more pronounced when oligonucleotides containing uncharged backbones are assayed, presumably because some of the interactions between the dye and the nucleic acid are ionic in nature. Finally, OliGreen reagent is not selective for detecting ss nucleic acids and also has significant fluorescence enhancement upon binding ds nucleic acids. The fluorescence excitation maximum for OliGreen reagent bound to nucleic acids is ~500 nm and the fluorescence emission maximum is ~520 nm-similar to fluorescein excitation and emission wavelengths.

Table 2

### Comparison of Dyes for Detecting Nucleic Acids in Electrophoretic Gels

DYE	SENSITIVITY*			
	ds DNA	RNA	ss DNA	short ss oligonucleotides
SYBR Green I stain	40-60 pg/band (300)	1 ng/band (300)	1 ng/band (300)	9 ng/band (300)
	10-20 pg/band (254)	200 pg/band (254)	200 pg/band (254)	1-2 ng/band (254)
		500 pg/band (300)	500 pg/band (300)	
SYBR Green II stain		100 pg/band (254)	100 pg/band (254)	
ethidium bromide	500 pg/band (300)	1.5 ng/band (300)	1.3 ng/band (300)	100 ng/band (300)

\* Sensitivity indicates the sensitivity limit obtained using Polaroid 667 black-and-white print film to photograph samples electrophoresed through nondenaturing 1% agarose/TBE minigels, through a SYBR Green gel photographic filter (for SYBR Green dye stained gels) or an ethidium bromide photographic filter or Wratten 25 filter (for ethidium bromide stained gels). The same sensitivity limits for SYBR Green II stain were also obtained for RNA electrophoresed on a 5% polyacrylamide/urea denaturing gel and for ds DNA electrophoresed on a 5% polyacrylamide nondenaturing gel, stained with SYBR Green I

dye. Each stain was used under its own optimal staining conditions and the best exposure (that showing the highest sensitivity) was scored. SYBR Green dye stained gels were photographed using 254 nm epillumination and 300 nm transillumination. Ethidium bromide stained gels were photographed using 300 nm transillumination. The illumination source wavelength in nm is indicated in parentheses following the sensitivity limit. A FOTO UV® /450 transilluminator (FotoDyne) was used for all ultraviolet illumination.

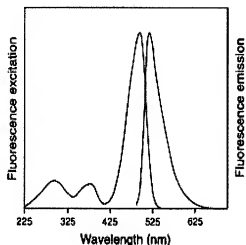


Figure 7. Fluorescence excitation and emission spectra of SYBR Green I nucleic acid stain bound to calf thymus DNA. The excitation spectrum was obtained by monitoring emission at 530 nm and the emission spectrum was obtained after excitation at 480 nm.

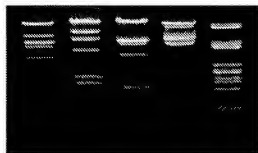


Figure 8. Bacteriophage Icl857 DNA prestained with a 1:10,000 dilution of SYBR Green I stain, then digested with restriction endonucleases and separated on a 1% agarose gel. Lanes contain DNA digested with (left to right) 1) EcoRI, 2) HindIII, 3) XhoI and EcoRI, 4) BamHI, and 5) PstI.

## Gel Staining

For detecting nucleic acids in gels, optimal dyes would be expected to exhibit high quantum yields, good molar extinction coefficients and fluorescence excitations at both ultraviolet and visible wavelengths, good fluorescence enhancements (greater than 100-fold), low intrinsic fluorescence, and fluorescence emission at wavelengths that can be readily captured by Polaroid® black-and-white print film, CCD image documentation systems, and commercially available laser scanners. Of the dyes screened for staining electrophoretic gels, SYBR Green I gel stain yielded the best sensitivity for detecting ds DNA and ss oligodeoxynucleotides, and SYBR Green II stain gave the best sensitivity for detecting RNA and longer ss nucleic acids (Fig. 6). Table 2 shows a comparison of the sensitivity limits obtained with these dyes and with ethidium bromide in parallel experiments. When bound to nucleic acids, both dyes have excitation maxima at ultraviolet and visible wavelengths (Fig. 7). SYBR Green I stain binds very tightly to ds DNA, allowing it to be used as an electrophoretic pre-stain<sup>60</sup> (Fig. 7), like the cyanine dimers discussed above. However, both SYBR Green I and SYBR Green II stain can be readily removed from DNA by ethanol precipitation, leaving the DNA available for subsequent cloning or other manipulation. SYBR Green I stain also does not interfere with restriction endonuclease digestion when present at standard gel staining concentrations<sup>61</sup> (Fig. 8). In addition, gels do not require destaining to achieve optimal sensitivity because unbound dye has negligible intrinsic fluorescence. Urea/polyacrylamide and formaldehyde/agarose RNA gels can be stained with SYBR Green II dye without washing out the denaturant, allowing gels to be photographed immediately after staining. Unlike silver staining, the SYBR Green gel stains do not produce shadow bands when lanes are overloaded; their dynamic range for nucleic acid detection is thus greater than that obtained with silver staining. Neither dye interferes with Southern or Northern blotting. Capillary transfer of nucleic acids to filter membranes is unaffected and, as long as 0.1–0.3% sodium dodecyl sulfate is included in the prehybridization or hybridization buffers, there is also no effect on

### hybridization efficiency.

The binding modes of the SYBR Green gel stains are unknown. However, in comparison to ethidium bromide, SYBR Green I stain exhibits a much lower level of mutagenicity in Ames tests-particularly on frameshift indicator strains-suggesting that the dye either does not intercalate or is efficiently displaced during replication.<sup>62</sup> In collaboration with Dr. Claire Berg at the University of Connecticut, we have also found that when SYBR Green I dye is used as a pre-stain, it appears to relax negatively supercoiled DNA, indicating that it has a strong effect on the DNA linking number. In addition, SYBR Green I stain complex formation with DNA is salt-dependent, thus the dye probably has some electrostatic interactions with nucleic acids.<sup>59</sup> It is not known if other dyes in this class have these same properties.

Both gel stains have been employed to develop sensitive bioanalytical assays. SYBR Green I nucleic acid gel stain has been used to develop a nonradioactive method for detecting hypervariable simple sequence repeats<sup>63</sup> and a sensitive gel-based assay for telomerase activity.<sup>64</sup> It has also been shown to provide much greater sensitivity for detecting reverse transcription PCR products than can be obtained with ethidium bromide,<sup>65</sup> and has been reported to provide a detection limit of 80 fg when analyzing ds DNA by capillary electrophoresis.<sup>66</sup> In the latter application, SYBR Green I stain compared very favorably with the cyanine monomer and dimer dyes discussed above, providing a broader dynamic range for DNA detection with greater linearity. In addition, it has been used to quantitate PCR products during DNA amplification using a rapid cycle thermocycler.<sup>67</sup> We also found that SYBR Green I stain provides a sensitive reagent for detecting bandshift or electrophoretic mobility shift products and is particularly useful for detecting complexes formed by DNA replication factors on closed, circular, ss DNA templates,<sup>68</sup> which cannot be readily labeled with radioactive probes. SYBR Green II RNA gel stain has been used to develop a solution quantitation assay for RNA<sup>69</sup> and a sensitive alternative method to silver staining for detecting SSCP products.<sup>70</sup>

## Summary

The properties and biological applications of a new series of unsymmetrical cyanine dyes are described. The new dyes provide extremely sensitive one-step methods for detecting and quantitating nucleic acids in gels and in solution. These procedures readily lend themselves to automation and high-throughput analysis, both because of their ease of application and because of their compatibility with commercially available detection instrumentation, including inexpensive ultraviolet epi- or transilluminators and Polaroid cameras or CCD-based image acquisition systems, laser-excited gel scanners, fluorescence microplate readers, and capillary electrophoresis systems. In addition, these dyes are now being used to develop sensitive assays for biologically important enzymes, such as reverse transcriptase, DNA polymerases, and telomerase, as well as to detect DNA amplification products and to quantitate small amounts of DNA isolated from precious sources, including human biological samples used for genomics or forensics analysis.

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